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Glucuronoyl esterases are active on the polymeric substrate methyl esterified glucuronoxylan

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ABSTRACT

Alkali extracted beechwood glucuronoxylan methyl ester prepared by esterification of 4-*O*-methyl-D-glucuronic acid side residues by methanol was found to serve as substrate of microbial glucuronoyl esterases from *Ruminococcus flavefaciens*, *Schizophyllum commune* and *Trichoderma reesei*. The enzymatic deesterification was monitored by ^{1}H NMR spectroscopy and evaluated on the basis of the decrease of the signal of the ester methyl group and increase of the signal of methanol. The results show for the first time the action of enzymes on polymeric substrate, which imitates more closely the natural substrate in plant cell walls than the low molecular mass artificial substrates used up to present.

1. Introduction

The discovery of the enzyme called glucuronoyl esterase in the cellulolytic system of the wood rotting fungus *Schizophyllum commune* using synthetic esters of uronic acids [1] suggested that the enzyme could have important biotechnological implications. There are reports that one of the covalent linkages between hemicellulose and lignin in plant cell walls are ester linkages between 4-*O*-methyl-D-glucuronic acid (MeGlcA) and lignin alcohols [2–13] which could be potential substrates of the esterase. After the genes coding for glucuronoyl esterase were found to be widely distributed in both prokaryotic and eukaryotic microorganisms, a new family of carbohydrate esterases, CE15, was established [14,15]. However, only a few CE15 enzymes have been characterized in terms of catalytic activity up to now [1,14,16–18], and only two members of the family have had their 3D structure determined [19–21]. To general disappointment, there is no report that would demonstrate the physiological role of glucuronoyl esterases in microbial degradation of plant cell walls or lignin–carbohydrate complexes. However, glucuronoyl esterases certainly play an important role in this process, since they appear to be inducible constituents of plant cell wall degrading enzyme systems, they are frequently bi- or multimodular enzymes containing carbohydrate binding modules (CBM) [14,17] and they are also parts of cellulosomes, like the enzyme from *Ruminococcus flavefaciens* [22,23]. The activity of glucuronoyl esterases was demonstrated only on low molecular mass artificial substrates [1,14,16–18]. The enzymes are active on synthetic alkyl and alkylaryl esters of MeGlcA and glucuronic acid [24–27], and also on their aryl α- or β-glycosides [24], which indicates that they could recognize as substrates also esters of β-glycosidically linked MeGlcA. Such residues occur in arabinogalactans [28], however, their esterification has not been reported. The enzymes were found to be inactive on similar esters of D-galacturonic acid which excludes their participation in enzymatic hydrolysis of pectin substances [24]. Cloning of microbial GE genes in plants, in order to find out more about their physiological role, had deteriorating effect on plant development in *Arabidopsis* [29], and led to changes in the cell wall composition in aspen [30].

In this work we provide the first experimental evidence that glucuronoyl esterases recognize methyl esters of MeGlcA as a part of high molecular mass polysaccharide. The methyl ester of beechwood glucuronoxylan was prepared by alkali-catalyzed esterification. The modified polysaccharide and its deesterification

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by enzymes was studied by NMR. We show that monitoring of the decrease of the ester signal or increase of the signal of methanol could be used as basis for an alternative assay of glucuronoyl esterase.

2. Materials and methods

2.1. Enzymes

Glucuronoyl esterase from *Trichoderma reesei* was a recombinant enzyme obtained by homologous cloning of its catalytic domain as described earlier [14]. *S. commune* glucuronoyl esterase was prepared by cloning in *Pichia pastoris* as reported [27]. Both enzymes were shown to be electrophoretically homogeneous in the original papers [14,27]. Glucuronoyl esterase from *R. flavefaciens* was a recombinant enzyme prepared by cloning of the carboxy-terminal domain of a multi-domain protein CesA [22,23] in *Escherichia coli* as follows: An 1.5 kb DNA band coding for CesA amino acid residues from 352 to 768 plus a Met coding codon at the beginning, *Nde*I and *Not*I restriction sites on the 5′ and 3′ ends, respectively, were synthesized by Mr. Gene GmbH (Regensburg, Germany). Cloning of the 1.5 kb DNA fragment into PET24b, transformation *E. coli* BL21 (DE3), induction for the target protein expression, and extraction of proteins from *E. coli* cells were done according to the Novagen pET system suggested protocols. Purification of the active enzyme was done using column chromatograph on an AKTA Protein Purification System and the enrichment of the active enzyme was traced by activity against 4-O-methyl-D-glucuronic acid methyl ester hydrolysis followed by thin layer chromatography [1]. Purity of the target enzyme was accessed using SDS–PAGE followed by Coomassie Brilliant Blue staining (Fig. S1).

2.2. Preparation of glucuronoxylan methyl ester and other substrates

Beechwood glucuronoxylan, isolated by alkaline extraction from delignified holocellulose [31], was converted to its methyl ester a by modification of the procedure previously used for methyl esterification of galacturonic acid residues of pectin [32]. Briefly, glucuronoxylan was dissolved in water, converted into the tetrabutylammonium (TBA) salt using cation exchanger Amberlite IRN 77 (TBA^+ form) and lyophilized. The TBA salt (1000 mg) was dissolved in dry DMSO (6 ml) under stirring at 50 °C. After cooling, methyl iodide (1.8 ml) was added and the reaction mixture stirred for 2 days at 30 °C. After addition of 3 M NaCl (6 ml), the mixture was poured into acetone (600 ml). The formed precipitate was filtered, washed with acetone, suspended in deionized water and dialyzed. Lyophilization of the retentate afforded the esterified polysaccharide in about 90% yield from which only one third was found to be soluble in water. The water-soluble fraction of the glucuronoxylan methyl ester used as polymeric substrate of glucuronoyl esterases. Its average molecular weight, determined by high pressure gel permeation chromatography using two coupled HEMABIO columns (100 and 300, 8 × 250 mm) (Tessek, Praha, Czech Republic) and refractometric detection, was found to be 37.5kDa. The columns were eluted with 0.1 M aqueous solution of $NaNO_3$. The set of pullulans was used as standards. The polymeric character of glucuronoxylan methyl ester was also obvious from a TLC analysis which confirmed the absence of fragments shorter than decasaccharide. The structural features of the starting polysaccharide and its methyl ester is shown in Fig. 1. 4-Nitrophenyl 2-*O*-(methyl 4-*O*-methyl-D-glucopyranosyluronate)-β-D-xylopyranoside [33] was a generous gift from Dr. Ján Hirsch (Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia).

2.3. NMR measurements

NMR measurements were performed in D_2O at 25 °C on VNMRS 600 MHz Varian spectrometer equipped with HCN ^{13}C enhanced salt tolerant cold probe. Chemical shifts were referenced to internal standard 3-methyl silylpropionic acid sodium salt (TSP, δ 0 ppm). ^1H NMR spectra were acquired using presat sequence. Advanced techniques from Varian pulse sequence library of 2D homo- and hetero-correlated spectroscopy were used for the signal assignments.

2.4. Monitoring of glucuronoyl esterase action

The deesterification of glucuronoxylan methyl ester was monitored by ^1H NMR. 10 mg of the polysaccharide, twice evaporated from D_2O, was dissolved in 0.65 ml D_2O, the pH of the solution was adjusted to 6.0 with 0.2 M solution of deuterized sodium acetate (Aldrich Chemicals, USA) in D_2O. After recording the ^1H NMR spectrum of the starting polysaccharide, appropriate amount of tested glucuronoyl esterase lyophilized twice from D_2O was added and the spectra recorded in time intervals. Changes in signal intensity of the MeGlcA ester methyl group and MeOH in time course were monitored. The rate of deesterification was evaluated on the basis of signal heights. The dependence of the rate of

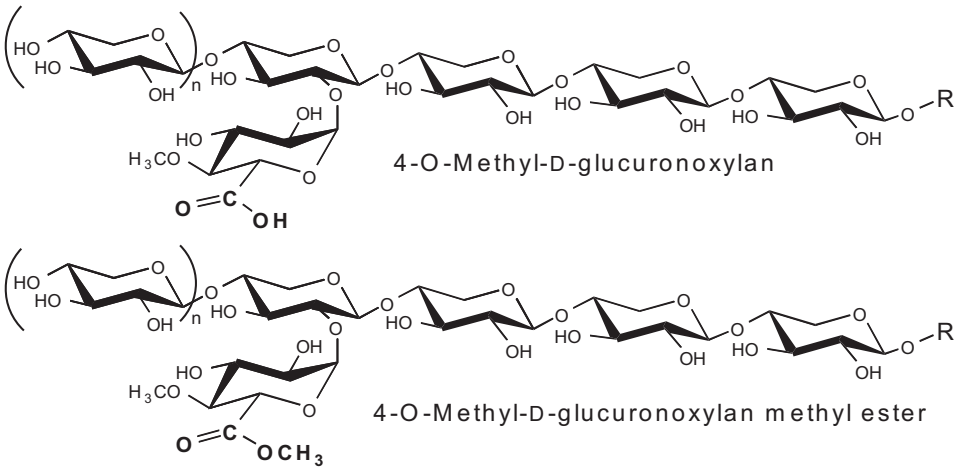

Fig. 1. Scheme of glucuronoxylan and its methyl ester.